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**UTILITY
PATENT APPLICATION
TRANSMITTAL**

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No. 4518US

First Inventor or Application Identifier Robert Hans Maeloen, Henrica Berendina Osk

Title See 1 in Addendum

Express Mail Label No. EL700256328US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO:Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

1. ☒ * Fee Transmittal Form (e.g., PTO/SB/17)
(Submit an original, and a duplicate for fee processing)
2. ☒ Specification [Total Pages 45]
(preferred arrangement set forth below)
- Descriptive title of the invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Microfiche Appendix
 - Background of the invention
 - Brief Summary of the invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets 4]
4. Oath or Declaration [Total Pages]
- a. ☐ Newly executed (original or copy)
 - b. ☐ Copy from a prior application (37 C.F.R. § 1.63(d))
(for continuation/divisional with Box 17 completed)
[Note Box 5 below]
 - i. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting
inventor(s) named in the prior application,
see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).
5. ☒ Incorporation By Reference (useable if Box 4b is checked)
The entire disclosure of the prior application, from which a
copy of the oath or declaration is supplied under Box 4b, is
considered to be part of the disclosure of the accompanying
application and is hereby incorporated by reference therein.

6. ☐ Microfiche Computer Program (Appendix)
7. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
- a. ☒ Computer Readable Copy
 - b. ☒ Paper Copy (identical to computer copy)
 - c. ☒ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 C.F.R. § 3.73(b) Statement ☐ Power of Attorney
(when there is an assignee)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
12. ☒ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
14. ☐ * Small Entity Statement(s) ☐ Statement filed in prior application,
(PTO/SB/09-12) Status still proper and desired
15. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)
16. ☐ Other:

* A new statement is required to be entitled to pay small entity fees, except
where one has been filed in a prior application and is being relied upon.17. If a **CONTINUING APPLICATION**, check appropriate box, and supply the requisite information below and in a preliminary amendment:

☐ Continuation ☐ Divisional ☒ Continuation-in-part (CIP) of prior application No. 09/274,048

Prior application information: Examiner A. Davenport Group / Art Unit: 1653

18. CORRESPONDENCE ADDRESS☒ Customer Number or Bar Code Labelor ☐ Correspondence address below

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Signature		Date	09/12/00

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Addendum

1. AN IMPROVED PEPTIDE, IMMUNOGENIC COMPOSITION AND VACCINE OR MEDICAL PREPARATION, A METHOD TO IMMUNISE ANIMALS AGAINST THE HORMONE LHRH, AND ANALOGS OF THE LHRH TANDEM REPEAT PEPTIDE AND THEIR USE AS VACCINE

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FEE TRANSMITTAL for FY 2000

Patent fees are subject to annual revision.
Small Entity payments must be supported by a small entity statement,
otherwise large entity fees must be paid. See Forms PTO/SB/09-12.
See 37 C.F.R. §§ 1.27 and 1.28.

TOTAL AMOUNT OF PAYMENT (\$ 894.00

Complete if Known

Application Number	
Filing Date	September 12, 2000
First Named Inventor	Robert Hans Meloen et al.
Examiner Name	
Group / Art Unit	
Attorney Docket No.	4518US

METHOD OF PAYMENT (check one)

1. ☒ The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to:

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description	Fee Paid
101 690	201 345	Utility filing fee	345
106 310	206 155	Design filing fee	0
107 480	207 240	Plant filing fee	0
108 690	208 345	Reissue filing fee	0
114 150	214 75	Provisional filing fee	0

SUBTOTAL (1) (\$ 345.00

2. EXTRA CLAIM FEES

Total Claims	Extra Claims	Fee from below	Fee Paid
81	-20** = 61	X 9 =	549
Independent Claims 3	-3** = 0	X 39 =	0
Multiple Dependent		0 =	0

**or number previously paid, if greater; For Reissues, see below

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description
103 18	203 9	Claims in excess of 20
102 78	202 39	Independent claims in excess of 3
104 260	204 130	Multiple dependent claim, if not paid
109 78	209 39	** Reissue independent claims over original patent
110 18	210 9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) (\$ 549.00

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description	Fee Paid
105 130	205 65	Surcharge - late filing fee or oath	
127 50	227 25	Surcharge - late provisional filing fee or cover sheet.	
139 130	139 130	Non-English specification	
147 2,520	147 2,520	For filing a request for reexamination	
112 920*	112 920*	Requesting publication of SIR prior to Examiner action	
113 1,840*	113 1,840*	Requesting publication of SIR after Examiner action	
115 110	215 55	Extension for reply within first month	
116 380	216 190	Extension for reply within second month	
117 870	217 435	Extension for reply within third month	
118 1,360	218 680	Extension for reply within fourth month	
128 1,850	228 925	Extension for reply within fifth month	
119 300	219 150	Notice of Appeal	
120 300	220 150	Filing a brief in support of an appeal	
121 260	221 130	Request for oral hearing	
138 1,510	138 1,510	Petition to institute a public use proceeding	
140 110	240 55	Petition to revive - unavoidable	
141 1,210	241 605	Petition to revive - unintentional	
142 1,210	242 605	Utility issue fee (or reissue)	
143 430	243 215	Design issue fee	
144 580	244 290	Plant issue fee	
122 130	122 130	Petitions to the Commissioner	
123 50	123 50	Petitions related to provisional applications	
126 240	126 240	Submission of Information Disclosure Stmt	
581 40	581 40	Recording each patent assignment per property (times number of properties)	
146 690	246 345	Filing a submission after final rejection (37 CFR § 1.129(a))	
149 690	249 345	For each additional invention to be examined (37 CFR § 1.129(b))	

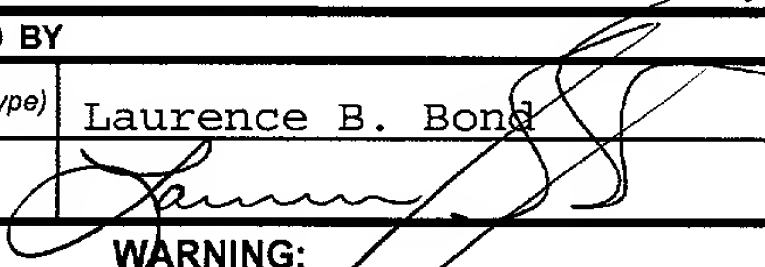
Other fee (specify) _____

Other fee (specify) _____

* Reduced by Basic Filing Fee Paid

SUBTOTAL (3) (\$

SUBMITTED BY

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Signature		Date	09/12/2000		

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Robert Hans MELOEN;
Hendrica OONK

Serial No.: To be assigned

Filed: September 12, 2000

For: AN IMPROVED PEPTIDE,
IMMUNOGENIC COMPOSITION AND
VACCINE OR MEDICAL PREPARATION,
A METHOD TO IMMUNISE ANIMALS
AGAINST THE HORMONE LHRH, AND
ANALOGS OF THE LHRH TANDEM
REPEAT PEPTIDE AND THEIR USE AS
VACCINE

Examiner: To be assigned

Group Art Unit: To be assigned

Attorney Docket No.: 4518US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EL700256328US

Date of Deposit with USPS: September 12, 2000

Person making Deposit: Jared Turner

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Sir:

Please revise the above-identified application as follows:

IN THE CLAIMS:

Please amend the claims as follows:

1. A peptide [that comprises]comprising a modified tandem GnRH decapeptide sequence [which allows for a]capable of inducing an immunogenic response that allows for discrimination between different types of GnRH[, preferably between GnRH-I and GnRH-II].

2. A peptide [that comprises]comprising a modified tandem GnRH decapeptide sequence which allows for a testosterone level that is essentially non-detectable after vaccination with the peptide in a suitable dosage.

3. A peptide according to claim 1[or 2 that comprises]comprising at least two coupled GnRH decapeptide sequences[, optionally coupled through a spacer], wherein at least one of the amino acids of said coupled GnRH decapeptide sequences is replaced by a different amino acid.

4. A peptide according to claim 1[, 2 or 3], wherein [the]said different amino acid is Ala.

5. A peptide according to claims 1[-4] wherein [the]said peptide is selected from the group consisting of:

SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7.

6. A peptide according to claims 1[-5] that is dimerised.

7. A peptide according to claim 6, conjugated with a carrier compound.

8. A peptide according to claim 7, wherein [the]said carrier compound is a protein.

9. A peptide according to claim [7 or]8, wherein [the]said carrier compound is ovalbumin.

10. A vaccine comprising a peptide in accordance [to any of]with claim[s] 1[-9].
11. A vaccine according to claim 10, [additionally]further comprising an adjuvant.
12. A vaccine according to claim 11, wherein [the]said adjuvant is an oil phase of an emulsion selected from the group consisting of a water-in-oil emulsion [or]and a double oil emulsion.

Note: in the originally filed claim set, two claims were numbered 13. Please amend the first claims 13 as follows:

13. A method for vaccinating a mammal against GnRH-I with a vaccine according to [claims]claim 10[-12].
14. A method for vaccinating a mammal according to claim 13[or 14], wherein [the]said vaccine is administered in a single dose.
15. A vaccine according to [claims 10-12]claim 10 that is sufficiently active for administration in a single dose for the essential immunocastration of pigs.
16. A method to effect one or more reproductive or behavioral characteristics of a mammal, characterized in that said mammal is vaccinated in accordance with [claims]claim 13[-15].
17. A method for immunizing a mammal against GnRH,[preferably GnRH-I,] comprising vaccinating the mammal with a vaccine according to claim 16.

18. A method to immunocastrate a pig, characterized in that said pig is vaccinated in accordance with claim 17[or 18].

19. Antibodies against GnRH-II obtainable by a method comprising a step wherein an immuneresponse is elicited to a peptide according to [claims]claim 1[-9].

20. A vaccine against GnRH-II comprising a peptide according to [claims]claim 1[-9].

21. Composition for the treatment of prostate cancer comprising a peptide according to [claims] 1[-9].

22. Use of a peptide according to [claims]claim 1[-9] in the preparation of a pharmaceutical composition.

23. Use of a peptide as defined in [claims]claim 1[-9] for the preparation of a medicament for the treatment of prostate cancer.

24. Method for the treatment of prostate cancer comprising administration of a suitable dose of a composition comprising a peptide that elicits an at least an immunogenic response against GnRH-II.

Please add the following new claims:

- 5

62. A vaccine comprising a peptide in accordance with claim 3.
63. A vaccine comprising a peptide in accordance with claim 5.
64. A vaccine comprising a peptide in accordance with claim 6.
65. A vaccine comprising a peptide in accordance with claim 7.
66. A vaccine comprising a peptide in accordance with claim 9.
67. A method for vaccinating a mammal against GnRH-I with a vaccine according to claim 11.
68. A method for vaccinating a mammal against GnRH-I with a vaccine according to claim 12.
69. A vaccine according to claim 11 that is sufficiently active for administration in a single dose for the essential immunocastration of pigs.
70. A vaccine according to claim 12 that is sufficiently active for administration in a single dose for the essential immunocastration of pigs.
71. A method to effect one or more reproductive or behavioral characteristics of a mammal, characterized in that said mammal is vaccinated in accordance with claim 14.
72. A method to effect one or more reproductive or behavioral characteristics of a mammal, characterized in that said mammal is vaccinated in accordance with claim 15.

73. Antibodies against GnRH-II obtainable by a method comprising a step wherein an immuneresponse is elicited to a peptide according to claim 3.

74. Antibodies against GnRH-II obtainable by a method comprising a step wherein an immuneresponse is elicited to a peptide according to claim 5.

75. Antibodies against GnRH-II obtainable by a method comprising a step wherein an immuneresponse is elicited to a peptide according to claim 6.

76. Antibodies against GnRH-II obtainable by a method comprising a step wherein an immuneresponse is elicited to a peptide according to claim 7.

77. Antibodies against GnRH-II obtainable by a method comprising a step wherein an immuneresponse is elicited to a peptide according to claim 9.

78. A vaccine against GnRH-II comprising a peptide according to claim 5.

79. Composition for the treatment of prostate cancer comprising a peptide according to claim 5.

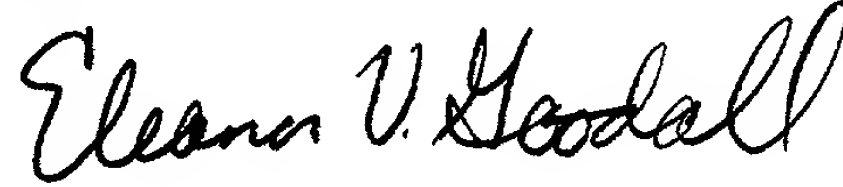
80. Use of a peptide according to claim 5 in the preparation of a pharmaceutical composition.

81. Use of a peptide as defined in claim 5 for the preparation of a medicament for the treatment of prostate cancer.

REMARKS

No new matter has been added. The Applicants request entry of the foregoing amendment prior to examination of the application on the merits.

Respectfully submitted,



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Date: September 12, 2000

ACT/EVG/dh

N:\2183\4518\Preliminary Amendment.wpd

PATENT
Attorney Docket 4518US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EL700256328US

Date of Deposit with USPS: September 12, 2000

Person making Deposit: Jared Turner

APPLICATION FOR LETTERS PATENT

for

**AN IMPROVED PEPTIDE, IMMUNOGENIC COMPOSITION AND VACCINE OR
MEDICAL PREPARATION, A METHOD TO IMMUNISE ANIMALS AGAINST THE
HORMONE LHRH, AND ANALOGS OF THE LHRH TANDEM REPEAT PEPTIDE
AND THEIR USE AS VACCINE**

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or the corresponding one letter code where pE is pyroglutamic acid and # is amide:

pE H W S Y G L R P G#.

GnRH-I acts at the hypophysis to cause an increase in release of biologically active Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH) in the blood, which in turn stimulate the development of the testes in the growing male animal and the synthesis of male steroids. In the growing female animal the development of the ovaries is stimulated, as is the development of follicles within the ovary, synthesis of female steroids, and ovulation.

It is known that GnRH-I, if coupled to a carrier protein, can be used to vaccinate animals. Such a vaccination can be carried out for various reasons, all of which are connected with the natural function of the GnRH-I. As is known, a drastic reduction of LH and/or FSH in the blood inhibits the production of male steroids or androgens and sperm in the testis of the male and the formation of female steroids or progestagens and estrogens and follicle maturation in the ovary of the female. Such a reduction in the amounts of androgens, progestagens and estrogens in the blood, to a level comparable to that obtainable by removing the testes or ovaries via castration, can be achieved by effective immunization of the animal against GnRH-I. In male animals, in many cases the testes then appear to develop slowly or not at all, with no synthesis of androgens (male steroid hormones) and no formation of spermatozoa. In female animals the activity of the ovaria appears to diminish, with no synthesis of estrogens and progestagens (female steroid hormones), and inhibition of ripening of follicles and ovulation.

Recently it was reported that a second form of GnRH (GnRH-II) is present in primate brain (Lescheid et al. Endocrinol. 138 (1997) 5618-5629) and a gene for this second GnRH molecule was cloned from a human genomic library (GnRH-II, (SEQ ID NO: 2) (White et al. PNAS USA 95 (1998) 305-309). Mammalian GnRH-I (SEQ ID NO 1) is hardly expressed outside the brain. A few exceptions are known in this respect. GnRH I is present in the endometrium of women with a menstrual cycle (Casan et al. Fertil. Sateril. 1998, 70, 102-106) and is expressed during pregnancy in the human placenta (Kelly et al. DNA cell Biol. 1991, 10, 411-421). GnRH mRNA was found in

ovary, testis, thymus, placenta and hypothalamus of the rat (Oikawa et al.,
Endocrinology, 1990, 127, 2350-2356). Expression of GnRH was detected in immune
tissue (spleen, thymus and lymphocytes) of pigs (Weesner et al., Life Sci, 1997, 61,
1643-1649).

5 GnRH-II is expressed in many tissues outside the brain, and is found in especially
high concentrations in the kidneys, bone marrow and prostate. The presence of GnRH-II
in diverse tissues other than the brain suggests that GnRH-II may have multiple
functions. In addition, the strictly conserved structure of the GnRH-II peptide throughout
diverse vertebrate species suggests that this neuropeptide possesses vital bioactivities.
10 Until now, however, the functions of GnRH-II have been practically unknown. Several
types of differentiated lymphocytes, such as T- and B-lymphocytes and mast cells,
produce GnRH and GnRH-like peptides. Significant numbers of the latter cell type are
present in kidney, bone marrow and prostate, perhaps contributing to the high GnRH-II
expression in these tissues. GnRH II seems less involved in reproduction as compared to
15 GnRH-I. In the hypogonadal mouse, mouse which lack the GnRH-I gene, GnRH-II
producing cells are present in the same distribution as in normal mouse, but this is not
sufficient to cause normal gonadal development in these mouse (Chen et al. FEBS Letters
435 (1998) 199-203). However macaques in luteal phase of the menstrual cycle showed
a marked increase in plasma luteinizing hormone concentrations after intravenous
20 administration of GnRH-II, but this increase could not be induced during the mid
follicular phase (Lescheid et al. Endocrinol. 138 (1997) 5618-5629.)

BRIEF SUMMARY OF THE INVENTION

25 The invention now provides the insight that by providing peptide sequences that
allow discrimination between the different types of GnRH, more adequate and efficient
use can be made of the variation or difference in immunological response to the different
types of GnRH. More particularly, the invention provides the insight that improvements
in the efficacy and selectivity of the vaccines against GnRH-I can be achieved.

30 Immunization against GnRH-I is effective in neutralizing GnRH-I and results in
reduced gonadotropin levels and blocking of gonadal steroid synthesis. However,

nothing is known about any physiological effects of the antibodies raised against GnRH-I on the function of GnRH-II. As GnRH-II is mainly synthesized and secreted in the kidneys antibodies raised against GnRH-I that cross-react with GnRH-II may affect kidney function. To obviate possible side effects of GnRH-I immunization on kidney function it would be desirable to direct the antigenic response of an immunocastration vaccine specifically toward GnRH-I and to avoid possible harmful side-effects due to neutralization of non-gonadal GnRH-II.

If the reproductive capacity alone, often with its accompanying sexual behavior, of a species needs to be annulled, it would be preferred to aim at an immunocastration vaccine specifically neutralizing GnRH-I. Hence the desire to come to a selective immunization against Gonadotropine Releasing Hormone(s), preferably selective against GnRH-I.

In veterinary medicine, 100% effective immunization against GnRH-I could be used for the sterilization of, e.g., small domestic animals such as male and female cats and dogs, or for the treatment of aggressiveness in male dogs and bulls, simply by vaccination instead of by drastic surgery such as castration or ovariectomy. Other conceivable reasons for immunization against GnRH-I are to prevent heat in female animals, such as dogs, cats and cows, and restlessness in male animals being fattened for slaughter.

In human health care, immunization against GnRH-I and/or GnRH-II can be used in the treatment of prostate cancer and breast cancer and, if required, in the treatment of some forms of pituitary carcinoma. In the case of prostate cancer it might be more desirable to neutralize both GnRH-I and GnRH-II, as the latter isoform is also highly expressed in prostate tissue.

Another use of a vaccine against GnRH-I is in the field of stock breeding, particularly the fattening of pigs for slaughter. The meat of male, sexually mature pigs (boars) has a typical odor, the so-called boar taint or boar odor. In the testes of the sexually mature pig, many C19-delta-16 steroids are formed which are stored in the fat tissue of the animal (Patterson, J. Sci. Food Agric. 19, 31-38 (1968); Brooks en Pearson, J. Anim. Sci. 62, 632-645 (1986); Claus, Zeitschrift. Tierzüchtg. Züchtungsbiol. 93,

38-47 (1976); Claus, *Acta Endocrinol. (Copenh.)* 91, Suppl. 225, 432-433 (1979)). These steroids are mainly responsible for the formation of the disagreeable urine-like odor when the meat is heated (Fuchs, *Swedish J. Agric. Res.* 1, 233-237 (1971); Bonneau, *Livest. Prod. Sci.* 9, 687-705 (1982)). Owing to this unpleasant odor, meat of male sexually mature pigs is generally unsuitable for consumption and unfit for export. Because about 10% of the male slaughter pigs are already sexually mature before the slaughter time, this potentially entails a great loss for the pig farming industry.

In order to control and prevent these losses, nearly all male piglets are castrated when they are young, with a surgical procedure that is generally executed without any form of anaesthesia. Apart from the animal unfriendly aspect of such a castration, castration also leads to infections, growth inhibition, and a final carcass quality inferior to that of an intact animal, at least as long as that intact animal has not yet developed boar taint (Walstra, *Livest. Prod. Sci.* 1, 187-96 (1974)).

An animal friendly alternative consists in the reduction of the GnRH-I concentration in the pig pituitary by means of immunization against GnRH-I, the so-called immunocastration. This reduction in GnRH-I levels leads to a reduction in the concentrations of biologically active FSH and LH, which in turn will inhibit development of the testes in the growing animals and inhibit the synthesis of testicular steroids, including androstenone, testosterone and estrogens. This method prevents the occurrence of boar taint in male pigs at slaughter time and makes surgical castration unnecessary as androstenone levels are reduced to levels low or undetectable (Oonk et al., 1995, *Livestock production Science* 42, 63-71).

A strict requirement for an acceptable vaccine against boar taint is that in almost all pigs development of the testes is delayed to such an extent that boar taint will not have occurred at the time of slaughter, and that in the case that the vaccine does not reduce testis development in an animal, this can be easily detected in a too large testis size in comparison to successfully immunocastrated pigs.

In the existing literature and previous patent applications regarding the anti-fertility properties of vaccines against GnRH-I, the results of vaccinations often appear to be variable. In most of the described studies, either a small percentage of the vaccinated

animals do not respond to the vaccination, or large doses of vaccine, multiple vaccinations or commercially unacceptable adjuvants are needed to produce the desired effect (Hoskinson et al., 1990, Austr. J. Biotech. 4, 166-170; Falvo et al.,(1986) J. Anim. Sci.63:986-994; Clarke et al., 1998, Endocrinology 139, 2007-2014; Adams T.E. and B.M. Adams, Feedlot performance of steers and bulls active immunized against Gonadotropine-Releasing Hormone, J. Anim. Sci. 1992, 70: 1691-1698; Brown et al., Immunization of sheep against GnRH-I early in life: effects of reproductive function and hormones in rams, Journal of reproduction and Fertility (1994) 101, 15-21; Ferro et al., Immunological castration using a Gonadotropine-releasing Hormone analogue conjugated to PPD, Food and agricultural immunology, 1995, 7, 259-272; U.S. patent 4,608,251; Int. patent appl. WO 88/05308).

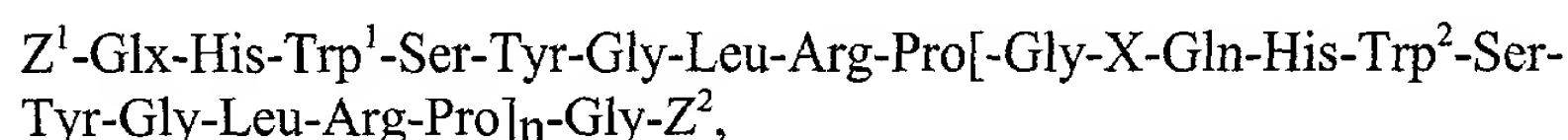
Some studies suggested an efficacy of 100% of a vaccine against GnRH-I, but the vaccine was not tested in a large number of animals (Ladd et al. (1994), Development of an antifertility vaccine for pets based on active immunization against Luteinizing Hormone releasing hormone, Biology of Reproduction 51, 1076-1083 ; J.G. Manns and S.R. Robbins (1997). Prevention of boar taint with a recombinant based GnRH vaccine, In: Boar taint in entire male pigs, Proceedings of a meeting of the EAAP working group "Production and Utilisation of Meat from Entire Male Pigs", EAAP Publication No. 92, 137-140;); other studies report the efficacy of the vaccine as the mean value of the treated animals, since individual values did not show a clear difference between immunized and untreated controls (Bonneau et al., J. Anim. Sci. 72, 14-20 (1994); Hennesy et al.,1997. Elimination of boar taint: a commercial boar taint vaccine for male pigs. In: Bonneau, M., Lundström, K. and Malmfors, B. (Eds.), Boar taint in entire male pigs. Wageningen Pers, Wageningen, EAAP Publication No. 92., 141-145).

The difficulty in preparing this type of vaccines probably is caused by the phenomenon of tolerance. Self substances such as hormones are not recognised, as foreign but rather are tolerated by the immune system. Normally no antibodies are elicited against self substances. In order for a vaccine to be successful, it must be sufficiently foreign. Only when the vaccine is foreign enough will the immune system not tolerate the vaccine and the production of antibodies be induced. Conversely,

however, the antibodies must still be capable of recognizing the hormone, and thus the vaccine cannot be too 'foreign'.

As these conditions appear to be mutually exclusive, it was not certain, until recently, if such substances could be prepared at all. One attempt to produce GnRH-like peptide vaccines consisted of the replacement of Gly at position 6 of the GnRH-I decapeptide by a dextrorotary amino acid (D-Tryp; Chaffaux et al., *Recueil de Médecine Vétérinaire* 161 (2), 133-145, 1985). It was, however, demonstrated that a vaccine preparation containing this modified GnRH-peptide performed even less well than the normal GnRH-I decapeptide (European Patent application-464,124 A).

Recently, we have shown definitively that it is possible to elicit an effective antibody response in all individuals vaccinated against GnRH-I (Meloan et al., *Vaccine* 12, 741-746 (1994)). In these experiments pigs were vaccinated twice with an GnRH-I vaccine that departs from the classical type of GnRH-I vaccine (GnRH-I coupled to a carrier protein, in Freund's adjuvant), namely the tandem-GnRH-I vaccine (European patent nr. 0464124). In this publication a peptide is described which is characterized in that it comprises at least 2 GnRH-I sequences in tandem (SEQ ID NO: 3) according to the general formula



in which amino acids are designated according to the three-letter code, Trp^1 and Trp^2 are tryptophan (Trp) or formylated tryptophan (N(indole)-formyl-tryptophan), n is a number having a value of at least 1, X is either a direct bond or a spacer group between the amino acids Gly and Gln, $Z^1\text{-Glx}$ is either pGlu (pyroglutamic acid) or Gln having attached thereto a tail comprising one or more additional amino acids, and Gly-Z^2 is either Gly- NH_2 or Gly having attached thereto a tail comprising one or more additional amino acids. In this general formula, X may be a direct bond between the amino acids glycine and glutamine, i.e. these amino acids are interconnected directly without an intermediate link (via the normal peptide bond). The tandem-GnRH-I vaccine invention also comprises peptides in which the GnRH-I sequences are interconnected via spacers. The nature of the spacer group may vary greatly, from one or more amino acids to a shorter or

longer hydrocarbon chain and other compound groups or molecules. In the above general formula, Z¹-Glx preferably stands for pGlu (pyroglutamic acid), but can also stand for Gln having attached thereto a tail comprising one or more additional amino acids, e.g., to be used for coupling of the peptide to a carrier protein. In the above
5 general formula, Gly-Z² stands for, e.g., Gly-NH₂, or Gly having attached thereto a tail comprising one or more additional amino acids, e.g., to be used for coupling of the peptide to a carrier protein. Preferably, Gly-Z² stands for Gly-Cys-NH₂, the C terminal cysteine being added in connection with a possible coupling of the peptide to a carrier protein.

10 From WO 96/40755 it is known that the tandem-dimer principle applied to a variant of the GnRH-I molecule resulted in a vaccine that was highly effective in several mild adjuvants, namely Specol and a double oil emulsion, and was also effective in low doses. In this case, the variant of the GnRH-I molecule was formed by substitution of the sixth amino acid Gly of the decapeptide by a dextrorotatory (D-) amino acid, D-Lys,
15 following which the resulting peptide was dimerised and coupled to a common carrier compound, ovalbumine. Thus, whereas a vaccine using D-amino acid substitutions of Gly at position 6 of the original and a single GnRH-I decapeptide with a D-amino acid decreased the immunogenicity as compared to the original GnRH-I sequence (Chaffaux et al., Recueil de Médecine Vétérinaire 161 (2), 133-145, 1985), such substitutions with a
20 D-amino acid applied to a tandem-dimer GnRH-I vaccine were able to generate even more immunogenic GnRH-I vaccine preparations. Nevertheless, the method for vaccination required a repeat dosage of the vaccine in order to be completely effective. The necessity of an additional booster dosage in order to achieve essentially 100% vaccination of mammals against GnRH-I is a disadvantage of the known peptides. We
25 also found that in certain cases use of the (D-Lys⁶)GnRH I tandem dimer (i.e. the GnRH I tandem dimer, with and without the D-Lys⁶ replacement) resulted in very low but still measurable amounts of testosterone, which is undesirable and a disadvantage of the (D-Lys⁶)GnRH I tandem dimer.

The present invention provides peptide sequences and that provide alternatives to the tandem D-Lys⁶ GnRH-I when applied in vaccines that result in vaccines that are effective for immunocastration.

5 An aspect of the present invention is the determination of the extent to which the amino acids in the tandem GnRH-I sequence can be varied while the resulting substituted tandem GnRH-I is still able to produce an immunogenic response to GnRH-I sufficient for immunocastration. Thus, the invention provides for the generation of a peptide sequence that can induce the production of antibodies against GnRH-I, which are also sufficiently competitive, both in amount and activity.

10 A further aspect of the present invention is to provide to a peptide sequence that selectively induces the production of antibodies against GnRH-I, while inducing little or no immune response towards GnRH-II. A preferred embodiment thereof is a peptide sequence that not only selectively induces the production of antibodies against GnRH-I but is also effective in immunocastration, while an immune response to GnRH-II is reduced or absent.

15 The present inventors have found that in the tandem GnRH-I peptide sequence various amino acids can be replaced, resulting in a decreasing resemblance to the self-hormone while at the same time retaining or even increasing the ability of the peptide to elicit GnRH-I binding antibodies. Also, certain replacements of amino acids in the
20 GnRH-I peptide sequence result in a selective immune response towards GnRH-I and to a reduced or absent immuneresponse towards GnRH-II.

In an aspect of the invention, certain modified tandem GnRH-I peptide sequences provide for vaccines that are not only capable of reducing testes growth in male animals but are also capable of essentially reducing testosterone levels to a degree that they can
25 not be determined by conventional techniques.

Further, vaccines prepared from these peptide-sequences express an activity that in most cases eliminated the need for the second booster immunization, as with the conventional D-Lys⁶ tandem GnRH-I, to achieve essentially 100% activity. An activity or efficacy of 100 % in the terms of the present invention is defined as a testosterone

level that is essentially undetectable with conventional techniques after a single vaccination.

One of the most notable features of the present invention is that antibodies raised by these alternative GnRH vaccines discriminate between GnRH-I and GnRH-II. Thus peptides according to the invention express an increased or retained activity against GnRH-I, while at the same time a reduced or absent immune response to GnRH-II is found. This allows for the development of peptides that express an inverse effect, that is, they express an increased or retained activity against GnRH-II while at the same time expressing a reduced or absent immune response against GnRH-I.

The invention relates in one aspect to a peptide that comprises a modified tandem GnRH-I decapeptide sequence whereby vaccination with the peptide in a suitable dosage allows for a testosterone level that is essentially non-detectable.

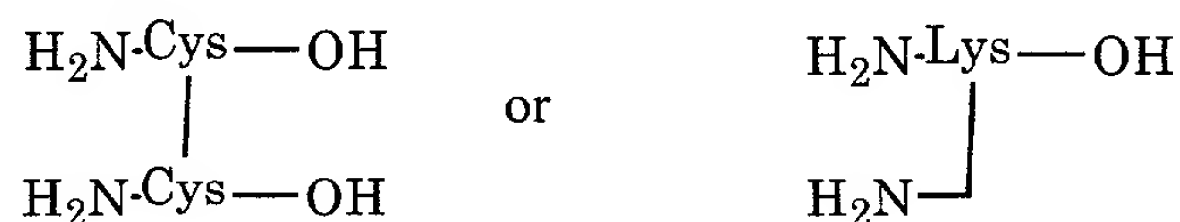
The invention also relates to a peptide that comprises at least two coupled GnRH-I decapeptide sequences, optionally coupled through a spacer, which allows for an immunogenic response that allows for the effective discrimination between GnRH-I and GnRH-II.

The peptides according to the invention are sufficiently like the hormone but at the same more 'foreign' to the immune system and have an increased capability to induce the production of antibodies directed against the hormone.

A feature of the invention is that individual tandem units can be dimerised to further enhance its immunogenicity without losing the possibility of coupling the peptide or peptide composition to a carrier compound protein.

The techniques for dimerisation and coupling of the tandem to a carrier similar to those described in WO 96/40755 may be used. It is also envisioned that peptides containing only a portion of the GnRH-I or II peptide sequences can be used in the present invention. Examples thereof are nonapeptides and undecapeptides.

The linkers for use in the peptides according to the invention can be selected from the linkers described elsewhere in this application or linkers such as



Linkers are used for coupling two or more dimerised peptide sequences. The amino acid which is used to replace the amino acid in the tandem peptide sequences is preferably an amino acid which is a relative simple one, such as alanine. In a preferred embodiment therefor the different amino acid is alanine. Other amino acids can also be used to replace the amino acid in the tandem decapeptide sequence. Preferably only conservative replacements are carried out. Conservative replacements are amino acid substitutions in which bulky amino acids are replaced by bulky amino acids, aromatic amino acids by aromatic amino acids etc. These concepts are well known to those skilled in the art.

Spacers can be placed between the peptides according to the invention. This allows for the formation of multimers. Suitable spacers are known in the art.

DESCRIPTION OF THE DRAWINGS

Figure 1: Competition for binding of iodinated GnRH with antiserum by GnRH (open circles), GnRH-II (solid circles), control peptide (solid squares) and no peptide (asterix). Serum is diluted 1/10000 and increasing peptide concentrations (0.25, 2.5 25 pmol) per well were added. In graphs A-C sera with increasing binding capacity for GnRH-II are shown. Horizontal axis: amount of peptide (pmol per well); vertical axis: Binding capacity (counts per minute).

Figure 2: Percentage of maximum binding of antiserum to iodinated GnRH after competition with GnRH-I (dark bars) or GnRH-II (light bars) for sera obtained after immunization with the peptides indicated in the left top corner of each graph. Antiserum obtained 3 weeks after boosterimmunisation was diluted 1:10000 and peptides for

competition were added in 250 nmol per liter. Horizontal axis : animal identification #; Vertical axis: percentage of maximum binding.

Figure 3: Percentage of maximum binding of antiserum to iodinated GnRH after competition with GnRH-I (dark bars) or GnRH-II (light bars) for antiserum against pE1A collected at 7 wpv (A), and at 10 wpv (B) and for antiserum against S4A collected at 7 wpv (C), and at 10 wpv (D). Antiserum obtained at 7 wpv and at 10 wpv was tested in an 1/2000 and 1/10000 dilution respectively. 25 pmol peptide for competition was added per well. Horizontal axis: animal identification #; Vertical axis: percentage of maximum binding.

Figure 4: Immunocastration efficacy score of several groups of pigs immunized with GnRH-tandem-dimer peptides (62 mg) conjugated to ovalbumine and Specol used as an adjuvant. The starting peptide was GnRH tandem (Cys-OH) dimer (abbreviated Cys-OH). Of this peptide all alanine scan peptides were used for immunization. Immunocastration efficacy was rated on a scale of 1 to 4 (1= immunocastration did not occur, 2 = a minority of the pigs was immunocastrated, 3 = a majority of the pigs was immunocastrated, 4 all pigs were immunocastrated).

DETAILED DESCRIPTION OF THE INVENTION

In a feature of the invention, the peptides according to the invention have the general formula given by (SEQ ID NO: 4), or by the following one-letter code:

pEHWSYkLRPGQHWSYkLRPGC#

In this general formula Q stands for Gln and may be preceded by X where X stands for a spacer. Several of these amino acids have been replaced with other amino acids. In the formulas the position of the substitution is depicted **bold** and underscored. Capital letters stand for Levorotatory amino acids, lower case letters stand for Dextrorotatory amino acids, e.g. K: L-Lys; k:D-Lys. Subsequently their immunogenic response has been determined when coupled to a carrier, generally ovalbumine, but other carriers such as KLH, BSA can be used.

It will be clear that the sequence of the GnRH-II peptide (SEQ ID NO: 2) determines which of the amino acids can be replaced in such manner that an effective

discrimination based on the immune response is still possible between the two sequences GnRH-I, and GnRH-II.

To determine whether antibodies raised by the GnRH vaccines according to the present invention discriminated between GnRH-I and GnRH-II, a GnRH antibody binding assay was performed in order to determine whether antibodies raised against GnRH-I-tandem dimer peptide or its alanine replacement analogs bind to GnRH-II or lack binding to GnRH-II. Serum dilutions were preincubated with either GnRH-I, GnRH-II, a control peptide or no peptide. Next iodinated GnRH-I was added to compete with the preincubated peptides for binding to the antibodies.

This procedure was performed for serum collected before and after booster immunization, as specificity of the antibodies may increase after booster immunization.

When the peptides according to the invention were used as the conjugate with ovalbumine (OVA-conjugate) and compared to controls, all showed effectiveness in the immunocastration of young male pigs. Comparison with the known G6k-GnRH tandem dimer OVA conjugate (see, Table 1), showed that the peptides according to the invention presented comparable or similar effectiveness even though their resemblance to the 'self'-hormone GnRH-I has decreased. The peptides according to the invention give an immunogenic response that allows for the effective discrimination between GnRH-I and GnRH-II. These peptides resulted in small testes and low testosterone levels. More specifically, the peptides expressing a low testes weight and a low testosterone level are R8A, G10A and S4A. The preferred peptides based on immunological selectivity between GnRH I and GnRH II are S4A and pE1A.

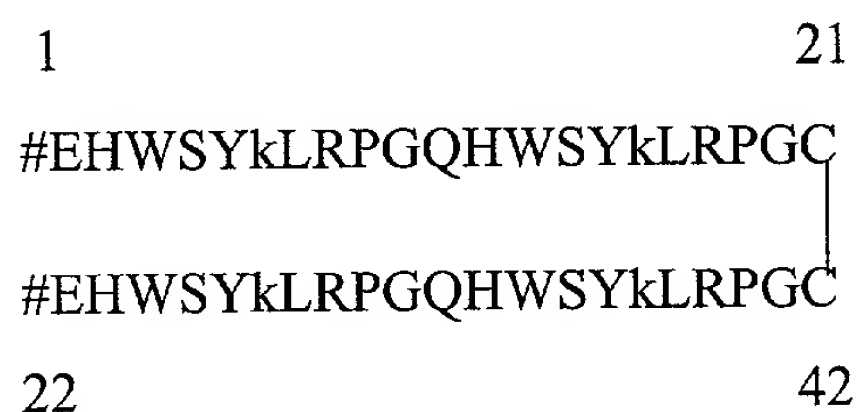
In a preferred embodiment, the peptide is selected from the group consisting of pEHWA_YkLRPGQHW_AYkLRPGC# (SEQ ID NO: 5), pEHWSYkL_APGQHWSYkL_APGC# (SEQ ID NO: 6) and pEHWSYkLRP_AQHWSYkLRP_AC# (SEQ ID NO: 7). It is more preferably selected from the group consisting of pEHWSYkL_APGQHWSYkL_APGC# (SEQ ID NO: 6); and pEHWSYkLRP_AQHWSYkLRP_AC# (SEQ ID NO: 7).

In the peptides according to the invention, dimerisation of the tandem units can for example take place via the carboxyl-terminus or via the amino-terminus. Two

tandem units may for instance be dimerised by means of a disulfide or thioether bridge.

To dimerise the tandem sequences the Cys at position 21 can be used, or Cys can be synthesized before the glutamic acid at position 1. Other methods to dimerise or multimerise the GnRH-tandem units can also be found in the prior art. If the Cys at position 21 is involved in the dimerisation and accordingly not available for coupling, it is likewise possible to use another amino acid of the tandem that can be coupled. If the dimerisation or multimerisation results in the loss of accessible sites where a carrier compound can be conjugated, it is sufficient to restrict the choice of replacement amino acids to an amino acid with an appropriate side chain. Such a replacing amino acid can be, for example, L or D-Lys, L or D-Glu or another amino acid containing a side chain that allows coupling to a carrier compound. Both L- and D- substitutions have been tested and found to have the same effect.

More in particular, an example of such a preferred peptide according to the invention, a D-Lys⁶-tandem-GnRH dimer (SEQ ID NO: 8) according to the following formula:



In this example of an embodiment of the invention it is possible to replace one of the amino acids of the tandem-dimer by another amino acid.

Other peptides or peptide sequences or coupled peptide sequences in which monomerised, dimerised or multimerised GnRH tandem units are present that contain amino acid substitutions are also part of the invention.

The invention further provides a composition which comprises a peptide brought into an immunogenic form. As a skilled worker knows, there are different methods of producing an immunogenic form of a substance which is in itself not immunogenic. One

possibility is to couple a peptide according to the invention to a suitable carrier protein. A suitable carrier protein is ovalbumin, KLH or BSA. In a tandem peptide, a cysteine at the – or C-terminus can be suitably used for a chemical coupling. In the tandem-dimer peptide, coupling can also be performed using the plain or the modified side chain of (D-) lysine, (D-) glutamine, or any other modified amino acid replacing amino acids of the peptide sequence. Suitable coupling methods and carrier proteins are well known to those of ordinary skill in the art.

According to the invention, there is preferred a composition which is characterized in that it comprises an immunogenic conjugate of a protein, such as ovalbumine, and a peptide or peptide composition.

A composition according to the invention can be used in the form of a vaccine. To this end the composition can be produced in a form that is suitable for administration. By administration of a vaccine according to the invention, an immunogenic response against GnRH is generated, preferably an immunogenic response against GnRH-I.

The invention therefore also provides a method of immunizing a mammal against GnRH-I, through vaccination of the mammal with a vaccine according to the invention. In a preferred embodiment, the invention provides a method of selectively immunizing a mammal against GnRH-I, with a vaccine according to the invention.

Of course, the vaccine preparation according to the invention can be combined with at least one immunoadjuvant. Suitable immunoadjuvants are known to those skilled in the art. A preferred adjuvant according to the invention can be Specol or a double oil emulsion, but other adjuvants that elicit no or only mild side-reactions can be used as well. The invention can be used in methods for immunizing individuals selected from a wide range of vertebrates, and particularly mammals, against GnRH-I. In a preferred embodiment of the invention, the vaccine can be administered in a single dose, which has the same efficacy as the presently known vaccines which must be administered in two-dose form. Immunization against GnRH-I, preferably selective, could for instance be used for the sterilization of, *e.g.*, small domestic animals such as male and female cats and dogs, or for the treatment of aggressiveness in male dogs and bulls. Other conceivable reasons of immunization against GnRH-I with the present invention are

preventing heat in female animals such as dogs, cats and cows, and preventing or treating restlessness in male animals being fattened for slaughter. In human health care, immunization against GnRH, preferably selective against either GnRH-I or GnRH-II, can be used in the treatment of prostate cancer and breast cancer and, if required, in the treatment of some forms of pituitary carcinoma.

A preferred embodiment is a method of improving the carcass quality of pigs, wherein the pigs are vaccinated with such a vaccine preparation according the invention. The invention is illustrated in the following experimental part.

I. Immunocastration of pigs

MATERIALS AND METHODS

Materials

Acetonitrile (ACN) was HPLC-S gradient grade, N-methylpyrrolidone (NMP), diisopropylethylamine (DIEA) dimethylformamide (DMF), trifluoroacetic acid (TFA) and piperidine were peptide synthesis grade and were all obtained from Biosolve (Valkenswaard, NL). N-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were obtained from Richelieu Biotechnologies Inc. (Hamon, Canada). Benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) was obtained from Novabiochem (Laufelfingen, Switzerland). Thioanisole (TA), ethanedithiol (EDT), dimethylsulfoxide (DMSO), pentane and dimethylaminopyridine (DMAP) were pro-analysis grade and were obtained from Merck (Darmstad, Germany). Diethylether was purified over a column of activated, basic aluminum oxide before use. Amino acid derivatives and resins were obtained from Bachem Feinchemicalien AG (Bubendorf, Switzerland).

Multiple peptide synthesis (MPS)

A Hamilton Microlab 2200 was programmed to deliver washing solvents and reagents to a rack with 40 individual 4 ml columns with filter, containing 30 mmol of resin for peptide synthesis. The columns were drained after each step by vacuum. The coupling cycle was based on Fmoc chemistry using double coupling steps:

pE = pyroglutamic acid; * = acetyl; # = amide; k = d-lysine; G6k = Gly on position 6 in the native GnRH sequence substituted by d-Lysine

Analytical HPLC

5 For analysis of peptides, we used a LC-MS (electrospray) system, which consists of two Waters pumps model 510, a Waters gradient controller model 680, a Waters WISP 712 autoinjector, and a Waters 991 photodiode array detector. The mass spectrometer was a Micromass Quattro II sq, which was used in positive ion mode. Products were analyzed in a linear gradient from 10% ACN/water with 0.05% TFA to 70% ACN/water
10 with 0.05% TFA in 30 min on a Waters Delta Pak C18-100Å (3.9x150 mm, 5 mm) column at 1 ml/min at 215 nm. All products were between 40-70 % pure according to the peak area.

Dimerisation

15 Crude products were dimerised by dissolving the products in 20% DMSO in water. The pH was adjusted to 5 - 6 with 1% NH_4HCO_3 , maintaining a clear solution. Correction of pH was done with 1% acetic acid. After stirring at room temperature for at least 5 h, the products were stored at -20°C until purification.

Preparative HPLC

20 Peptides purifications were carried out using a Waters Prep 4000 liquid chromatograph, equipped with a Waters RCM module with two PrepPak cartridges plus guard cartridge (40x210 mm or 25x210 mm) filled with delta-Pak C18-100Å (15 mm) material. In general, purifications were run using the same eluents as in analytical
25 HPLC, but at a gradient speed of 0.5% ACN/ min and a flow rate of 40 or 100 ml/ min. Peptides were detected at 215-230 nm using a Waters 486 spectrophotometer with a preparative cell. The peptides were lyophilised and purity was determined to be at least 90%.

Conjugate preparation

For conjugation via N-ethyl-N=-(3'-dimethylaminopropyl)carbodiimide hydrochloride ("EDC") to chicken egg albumin ("OVA") an equal weight of both the peptide and the carrier protein were dissolved separately in milliQ water and both solutions were mixed well. Next a 10-fold excess, based on weight equivalents, of EDC was dissolved in milliQ water. Subsequently, this solution was slowly added to the solution of peptide/OVA under continuous stirring, pH of this final solution is 5. After at least 6 h slowly shaking the product was dialyzed (MW cut-off 10,000) against a 300 times excess of milli Q water for two days. Water was refreshed twice a day. The loading was calculated from comparative amino acid analysis of the conjugate and the carrier protein. Amino acid analysis was performed using a Waters Pico-Tag system, after hydrolysis in a Pico-Tag workstation using 6N HCl at 150 °C for 1 h, and derivatisation with phenyl isothiocyanate.

According to the amino acid analysis the conjugates contained between 0.3 and 0.5 mg of peptide per mg of carrier protein, with exception of the P9A-G6k-GnRH-tandem-dimer conjugate which only contained 0.16 mg peptide per mg ovalbumine.

The G6k-GnRH-tandem-dimer OVA conjugate is abbreviated by G6k-TD. The conjugates with alanine substitutions are abbreviated by the amino acid in the native GnRH sequence that is replaced, its position and an A for the Alanine replacement. For example pE1A-G6k-GnRH-tandem-dimer OVA conjugate is pE1A.

Emulsion preparation

Specol (Special Oil Phase, ID-DLO, Lelystad, The Netherlands) consisting of two detergents in a light mineral oil was used as oil phase (Bokhout et al., 1981). The water-in-oil-(WIO)-emulsions were prepared using an Ultra Turrax (Janke and Kunkel, Staufen, Germany) with a stirring bar i 25 mm. The oil phase Specol (5 parts v/v) was brought into a 25 ml glass vessel and the water phase (4 parts v/v) consisting the conjugate in milli Q water was slowly added while the emulsion was stirred. After the water phase was added, the emulsion was stirred for half a minute at the same rotation speed (15000 rpm). Emulsions were stored overnight at 4°C to check stability and were administered to the animals the next day.

Animals

Male piglets, approximately 10 weeks of age, were involved in this experiment. The crossbred piglets were housed in half slatted pens and were given ad libitum access to feed and water.

Immunization

The piglets were randomly assigned to the treatments, 6 or 7 piglets per treatment. All animals were injected with 2 ml emulsion containing the dimerised tandem GnRH conjugates (i.e. 62 mg peptide) or an emulsion without antigen. Injections were administered intramuscularly in the neck at the start of the experiment (day 0) and 7 weeks later (7wpv). Thirteen weeks after initial immunization (13 wpv) all animals were slaughtered.

Measurements and blood sampling

Animals were weighed at day 0 and 7 and 13 wpv. Testis sizes were determined by measuring testis length with a vernier calipers at day 0, and 7, 10 and 13 weeks thereafter. Testis sizes were recorded as average of both testicles.

Blood samples were taken via puncture of the vena jugularis on the same days testis sizes were measured, and also 4 weeks after the initial immunization. Blood samples were kept overnight at 4°C and the next day serum was obtained by centrifugation (1500 g, 15 min). Serum samples were stored at -20°C until assayed.

Evaluation after slaughter

After slaughter testes were removed, dissected free of epididymes and weighed. Testes weights were recorded as average of both testicles.

Peptide antibodies

Antibodies to the peptides used for immunization were determined with an ELISA. Peptides were coated in the wells of a microtitreplate using glutardialdehyde

(GDA). GDA was coated to the surface of the wells by incubation with 0.2% GDA in 0.1 M phosphate buffer (pH 5) for 4 hours at room temperature . Plates were rinsed 3 times for 10 minutes with 0.1 M phosphate buffer (pH 8). One microgram peptide in 100 ml phosphate buffer (0.1 M, pH 8) was coated per well by incubating for 3 hours at 37°C.

5 Plates were stored at -20°C until used. Thawed plates were rinsed 3 times for 10 minutes with milli-Q water containing 8.2 g NaCl, 1.15 g Na₂HPO₄·2H₂O, 0.20 g NaH₂PO₄·2H₂O and 5 ml of a 10 % Tween 80 solution in water per litre water.

Serial serum dilutions of the antipeptide sera were allowed to react with the coated peptides for 1 hour at 25°C. After rinsing for 3 times 10 minutes goat-anti-pig IgG coupled to horseradish peroxidase (Dako, Glostrup, Denmark) was introduced as second antibody for 1 hour and ABTS (Boehringer, Mannheim, Germany) ((250 ml (2 g/100 ml) in 10 ml substrate buffer to which 20 ml H₂O₂ (3% solution)) was used as substrate. Absorption was measured at 405 nm.

15 *GnRH antibodies*

Antibodies to GnRH were determined as described by Meloen et al. (Vaccine 12, 741-746 (1994)). Serial dilutions of the pig antisera were allowed to bind to ¹²⁵I-GnRH. Titers are expressed as percentage binding of ¹²⁵I-GnRH at a given serum dilution.

20 *Testosterone*

Testosterone levels in serum were determined using a Coat-a-Count kit purchased from DPC laboratories, Los Angeles, CA.

RESULTS

25 *testis size and testis weight*

Seven weeks after the first immunization, immunocastration effects already could be observed by measuring testicle sizes. Three treatments (R8A, G10A and G6k-TD) showed hardly any increment (<10mm) of average testis size at time of booster. These treatments were successful with testis weights at slaughter being 70 grams or less. Other treatments that were effective in terms of low testes weights are pE1A and S4A, while in

group Y5A, L7A and P9A, 2, 1 and 1 animals did not respond to the immunization respectively (Table 2). Individual testis weight of immunocastrated animals did not exceed 70 grams, resulting in a clear difference between immunocastrated and not immunocastrated animals.

5

TABLE 2: Effectiveness of the different treatments according testis weight (g)

	treatment	individual testis weight(g)	testis weight (g)	number
			(median (range))	responders/ total number
10	G6k-TD	10,10,10,12,16,70	11 (10-70)	6 / 6
	pE1A	16,18,33,36,53,55,65	36 (16-65)	7 / 7
	S4A	10,14,15,36,40,43,69	36 (10-69)	7 / 7
	Y5A	15,19,34,44,69,210,235	44 (15-235)	5 / 7
	L7A	12,13,15,20,24,29,195	20 (12-195)	6 / 7
	R8A	10,10,12,15,17,19,19	15 (10-19)	7 / 7
	P9A	8,9,20,23,31,57,300	23 (8-300)	6 / 7
15	G10A	11,13,14,14,15,30,39	14 (11-39)	7 / 7
	Controls	150,173,204,206,236	204 (150-236)	0 / 5

Antibody response

Mean antibody titers against the peptides used for immunization are given in table 3. Mean antibody titer of pigs treated with H2A and P9A are lower than peptide antibody titer of other treatments.

Antibody titers of individual animals against the different peptides ranged from 2 to 4. Within a treatment animals that were not immunocastrated showed the lowest anti-peptide titer. Animals treated with H2A and W3A were not immunocastrated, but significant anti-peptide titers were present.

GnRH antibody binding percentages at 1/ 2000 serum dilution were undetectable or low in H2A and W3A groups. However at a 1/200 serum dilution antibodies were detectable in sera of all animals of group H2A and three animals of group W3A.

Animals with low average GnRH antibody titers were not immunocastrated. High antibody titers did always result in successful castrated animals. Testicle weight of animals with intermediate antibody titers varied from 15 to 300 gram.

Average GnRH antibody titers per treatment showed a clear relation with testis weight (median) per treatment (Table 3).

Testosterone

Testosterone levels of all successful treated animals were low and decreased after second immunization. However a majority (n=31) of the animals reflected a castration effect as early as four weeks after initial immunization by showing undetectable testosterone levels. Testis weight of these animals varied from 8–36 grams.

The majority of the animals were thus effectively immunized after the administration of a single dose.

Although testosterone levels at slaughter were low for all immunocastrated animals, two animals with testes weights of 65 and 70 grams had significant serum testosterone levels of 3.80 and 1.18 pmol/ml respectively.

The peptides that resulted in low testes weight combined with undetectable testosterone levels are considered the most effective peptides for the immunocastration of pigs. These peptides are S4A, R8A and G10A.

Testosterone levels at slaughter of pigs that were not immunocastrated animals varied between 0.46 and 48.91 pmol/ml.

TABLE 3: Effect of different treatments on mean testis weight, peptide antibody titer, GnRH antibody binding percentage and LH and testosterone levels in serum.

Treatment	testis weight (median range)	anti peptide titer at 10wpv	GnRH binding (average)	testosterone (pmol/ml) at 13 wpv median (range)
G6k-TD	11 (10-70)	3.32	17.2	n.d. (n.d.-1.18)
pE1A	36 (16-65)	3.24	15.7	n.d. (n.d.-3.80)

5	S4A	36 (10-69)	3.19	16.9	n.d. (n.d.)
	Y5A	44 (15-235)	2.92	11.3	n.d. (n.d.-9.44)
	L7A	20 (12-195)	3.52	17.6	n.d. (n.d.-48.91)
	R8A	15 (10-19)	3.34	17.7	n.d. (n.d.)
	P9A	23 (8-300)	2.62	15.8	n.d. (n.d-11.57)
	G10A	14 (11-39)	3.19	17.6	n.d. (n.d.)

wpv=weeks post vaccination, n.d.= not detectable

II. Replacement of D-amino acid with L-amino acid

10 D-lysine (k) at positions 6, 16, 27, and 37 in a tandem dimer is replaced by an L-lysine (K) and the resulting peptide tested (Table 4).

G6k-GnRH-tandem: pEHWSYkLRPGQHWSYkLRPGC# (SEQ ID NO: 9)

G6K-GnRH-tandem: pEHWSYKLRPGQHWSYKLRPGC# (SEQ ID NO: 14)

15 Table 4:

treatment	testis weight (g) median (range)	number responders/ total	anti peptide titer at 10 wpv	GnRH- binding (average)	testosterone in pmol/ml, at 13 wpv, (median)
G6k-TD	11 (10-70)	6/6	3.32	17.2	n.d.
G6K-TD	21 (9-175)	5/6	2.70	15.6	n.d.
Controls	204 (150-236)	0/5	n.d.	n.d.	2.05

20 n.d. : not detectable.

Replacement of D-lysine by L-lysine does not alter efficacy of the vaccine antigen.

III. Discrimination between GnRh-I and GnRH-II

25 An GnRH antibody binding competitive radioimmunoassay was performed in order to determine whether antibodies raised against G6k-GnRH-tandem (SEQ ID NO: 9) dimer peptide or its alanine replacement analogs bind to GnRH-II or lack binding to GnRH-II. Serum dilutions were preincubated with either GnRH-I, GnRH-II, a control

peptide or no peptide. Next iodinated GnRH-I was added to compete with the preincubated peptides for binding to the antibodies.

This procedure was performed for serum collected before and after booster immunization, as specificity of the antibodies may increase after booster immunization due to antibody maturation.

Materials and methods

Serum samples of the 10 wpv bleeding (3 weeks post booster immunization) were diluted 1/10000 in PBS with 0.4 % BSA (dilution buffer). Fifty μ l serum dilution was put in microwell plates and 25 μ l peptide solution (0, 0.25, 2.5 or 25 pmol peptide in dilution buffer per well) was added. This mixture was allowed to incubate for 24 hours at 4°C. The next day 25 μ l iodinated GnRH (approximately 13000 cpm) was added and after overnight storage (4°C) unbound peptide was separated from bound peptide with charcoal. After centrifugation supernatant was separated, counted and the percentage iodinated GnRH bound to the antibodies was calculated.

Antibodies collected after the first immunization (7 wpv) were tested in a 1/2000 serum dilution and only one peptide dilution was tested (25 pmol/well).

Results

Binding of iodinated GnRH at a 1/10000 serum dilution was observed for the sera of all treatments except for H2A and W3A sera. Competition with GnRH-I resulted in a dramatic reduction of binding of iodinated GnRH to the antiserum (see Figure 1). At the sera dilution we tested, inhibition of binding of iodinated GnRH by GnRH-I was dose dependent for most sera. As expected, none of the antisera bound to the control peptide.

Competition by GnRH-II for antibody binding to iodinated GnRH resulted in a reduced binding in all sera of R8A and G10A treatments, in most of the sera of treatments P9A and TDK and in half of the sera of treatments Y5A and L7A (see Figure 2). However, antibodies of substantially all animals of the pE1A and the S4A group completely lacked binding to GnRH II, and were thus specific for GnRH I.

The antibody binding results obtained with the pre-booster sera were different from the 10 wpv sera. Antisera of the pE1A and S4A treatments have been tested for their ability to recognize GnRH-I or GnRH-II (see Figure 3). For both peptides 10 wpv sera from 6 out of 7 animals did not bind GnRH-II. Of the pE1A sera obtained before
5 booster immunization 4 out of 7 animals showed binding to GnRH-II. Two sera did not recognize GnRH-II and one serum showed no binding to iodinated GnRH. The results of the S4A pre-booster sera are contradictory to the results of the 10 wpv sera. The pre-booster sera of all animals of the S4A group did recognize GnRH-II, with the inhibition of binding capacity of iodinated GnRH being similar for both GnRH-I and GnRH-II.

SEQUENCE LISTING

<110> Meloen, Robert Hans

Oonk, Hendrica Berendina

<120> An Improved Peptide, Immunogenic Composition and Vaccine or Medical Preparation, a Method to Immunise Animals Against the Hormone LHRH, and Analogs of the LHRH Tandem Repeat Peptide and their Use as Vaccine

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Oonk, Hendrica Berendina
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CLAIMS

1. A peptide that comprises a modified tandem GnRH decapeptide sequence which allows for an immunogenic response that allows for discrimination between different types of GnRH, preferably between GnRH-I and GnRH-II.

5

2. A peptide that comprises a modified tandem GnRH decapeptide sequence which allows for a testosterone level that is essentially non-detectable after vaccination with the peptide in a suitable dosage.

10

3. A peptide according to claim 1 or 2 that comprises at least two coupled GnRH decapeptide sequences, optionally coupled through a spacer, wherein at least one of the amino acids is replaced by a different amino acid.

15

4. A peptide according to claim 1, 2 or 3, wherein the different amino acid is Ala.

20

5. A peptide according to claims 1-4 wherein the peptide is selected from the group consisting of:

pEHWAAYkLRPGQHWAYkLRPGC#, pEHWSYkLAPGQHWSYkLAPGC#,

and

pEHWSYkLRPAQHWSYkLRPAC#, preferably selected from the group consisting of pEHWSYkLAPGQHWSYkLAPGC#

and

pEHWSYkLRPAQHWSYkLRPAC#.

25

6. A peptide according to claims 1-5 that is dimerised or multimerised.

7. A peptide according to claim 6, conjugated with a carrier compound.

30

8. A peptide according to claim 7, wherein the carrier compound is a protein.

9. A peptide according to claim 7 or 8, wherein the carrier compound is ovalbumin.

10. A vaccine comprising a peptide in accordance to any of claims 1-9.

11. A vaccine according to claim 10, additionally comprising an adjuvant.

12. A vaccine according to claim 11, wherein the adjuvant is an oil phase of a water-in-oil emulsion or a double oil emulsion.

13. A method for vaccinating a mammal against GnRH-I with a vaccine according to claims 10-12.

13. A method according to claim 13, wherein the vaccine is a selective vaccine for vaccination against GnRH-I.

14. A method for vaccinating a mammal according to claim 13 or 14, wherein the vaccine is administered in a single dose.

15. A vaccine according to claims 10-12 that is sufficiently active for administration in a single dose for the essential immunocastration of pigs.

16. A method to effect one or more reproductive or behavioral characteristics of a mammal, characterized in that said mammal is vaccinated in accordance with claims 13-15.

17. A method for immunizing a mammal against GnRH, preferably GnRH-I, comprising vaccinating the mammal with a vaccine according to claim 16.

18. A method to immunocastrate a pig, characterized in that said pig is vaccinated in accordance with claim 17 or 18.

19. Antibodies against GnRH-II obtainable by a method comprising a step wherein an immuneresponse is elicited to a peptide according to claims 1-9.

20. A vaccine against GnRH-II comprising a peptide according to claims 1-9.

21. Composition for the treatment of prostate cancer comprising a peptide according to claims 1-9.

22. Use of a peptide according to claims 1-9 in the preparation of a pharmaceutical composition.

23. Use of a peptide as defined in claims 1-9 for the preparation of a medicament for the treatment of prostate cancer.

24. Method for the treatment of prostate cancer comprising administration of a suitable dose of a composition comprising a peptide that elicits an at least an immunogenic response against GnRH-II.

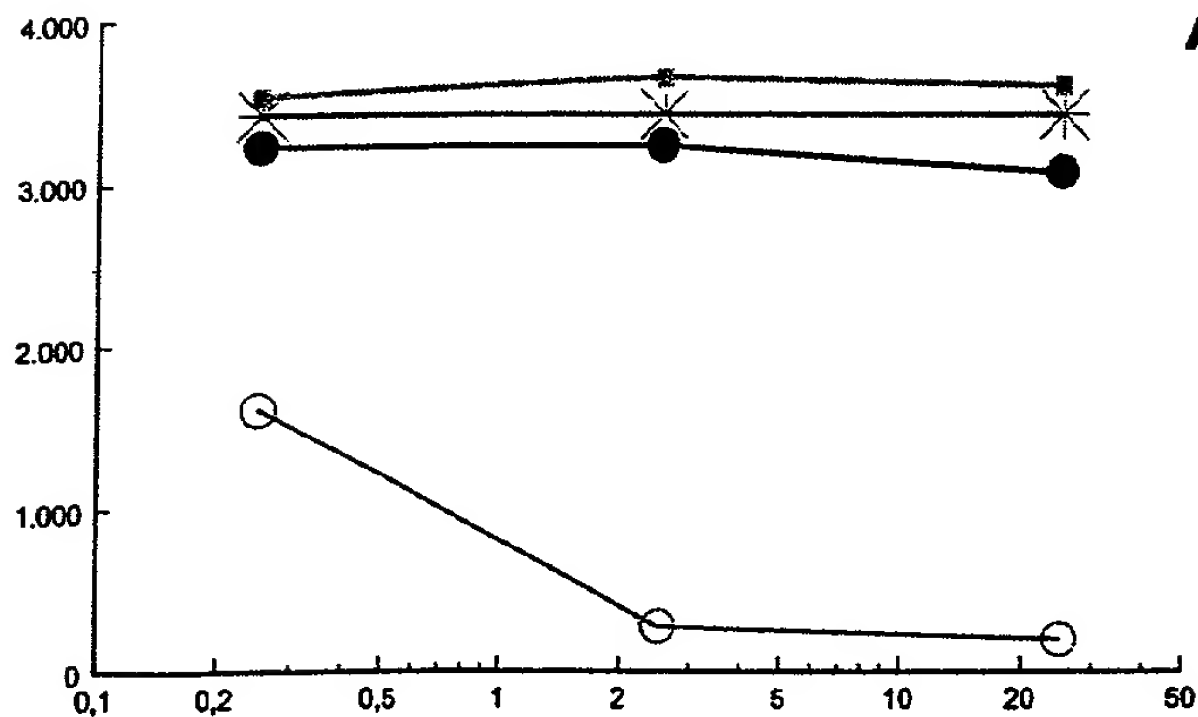
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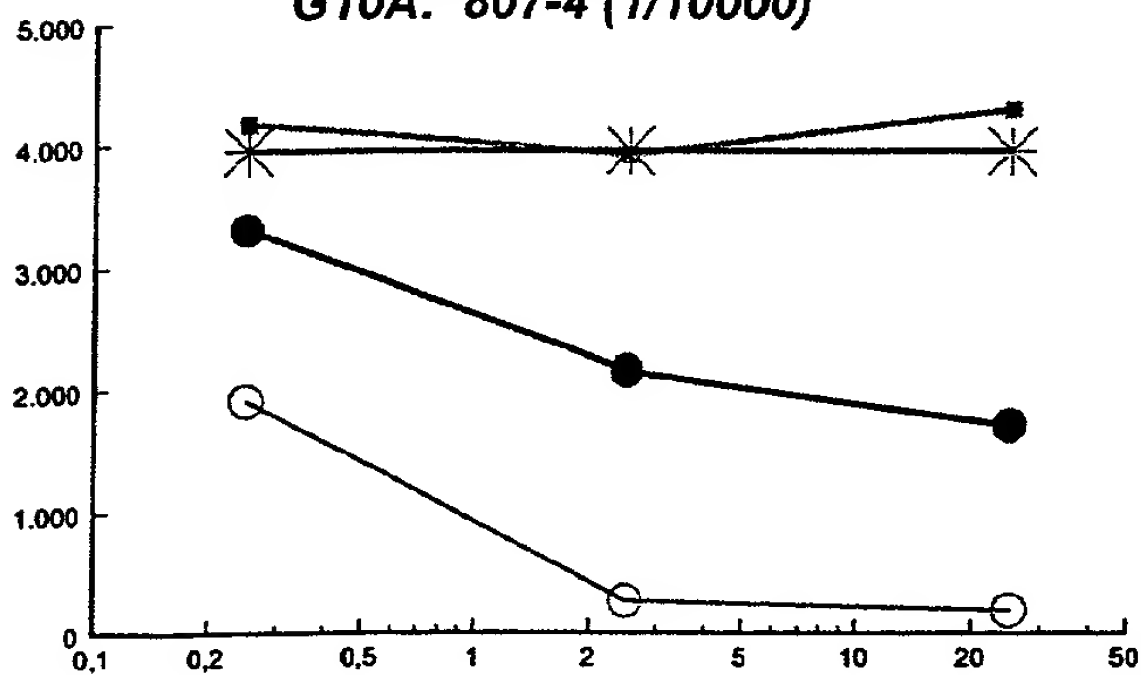
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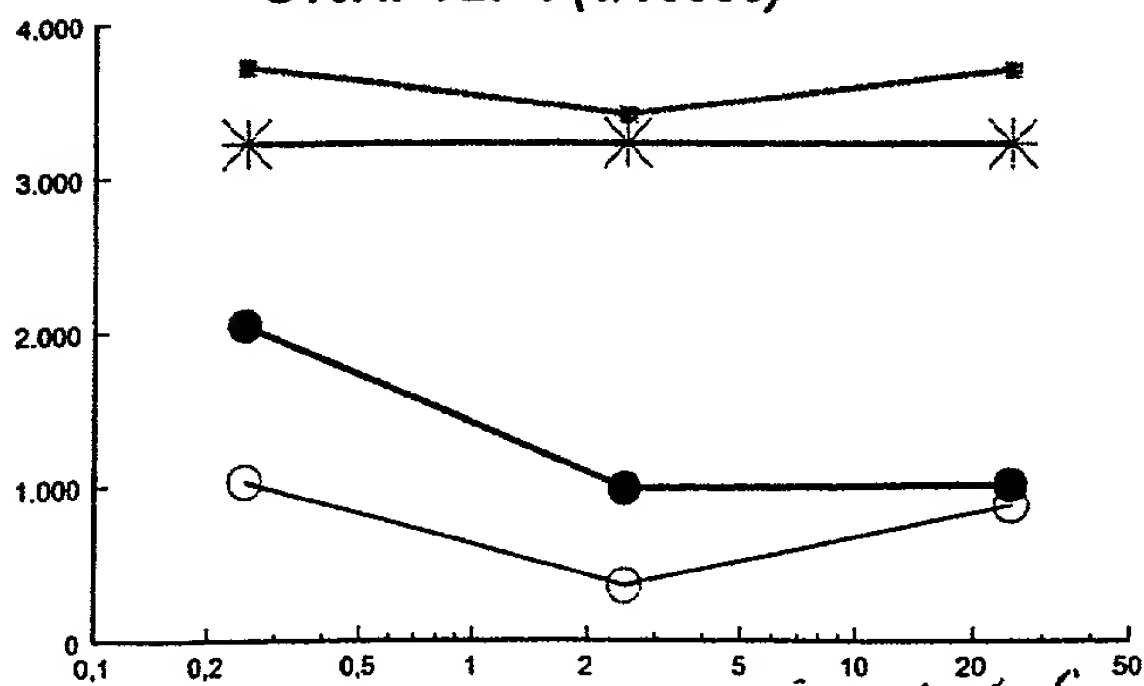


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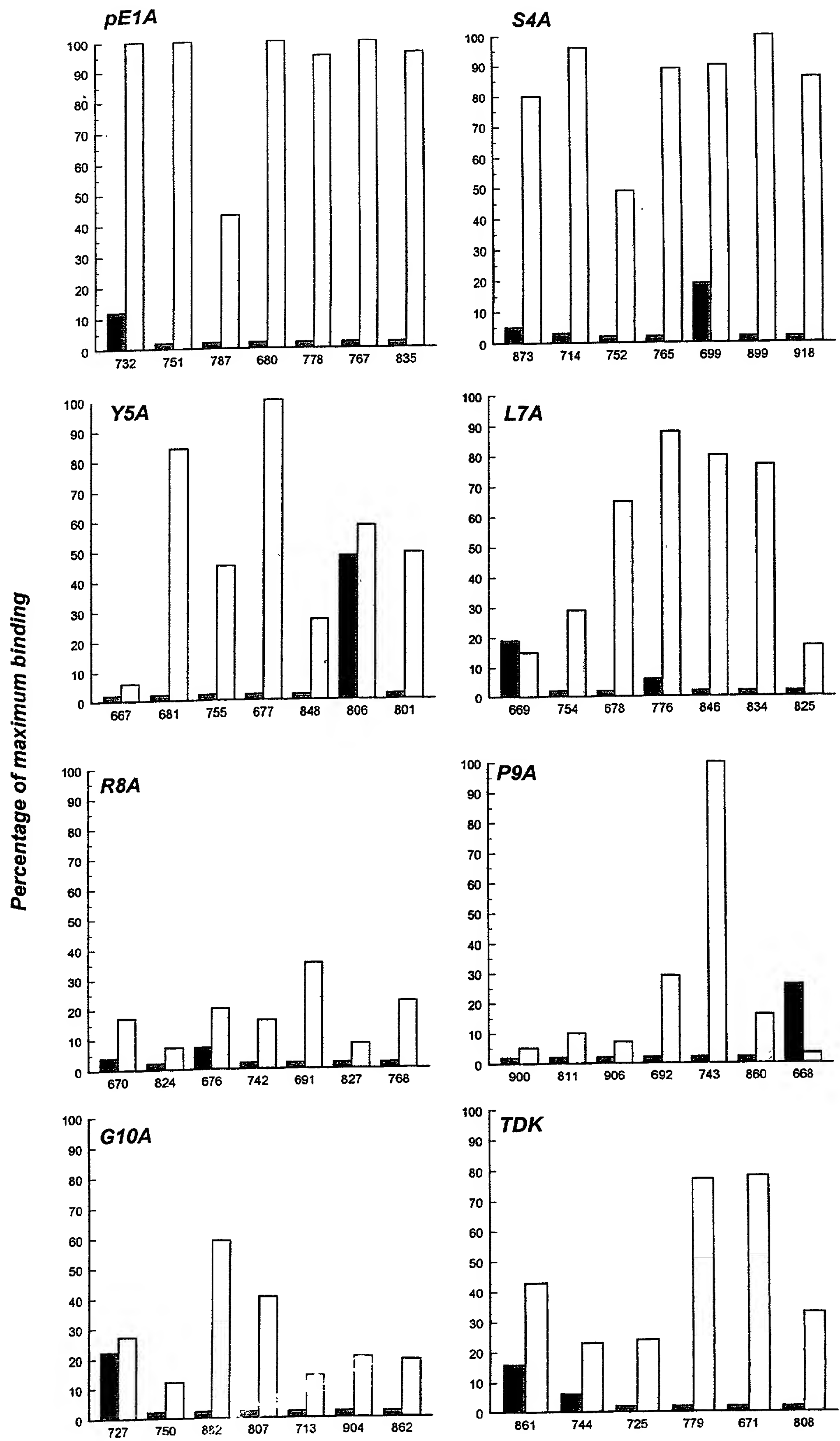


Figure 2

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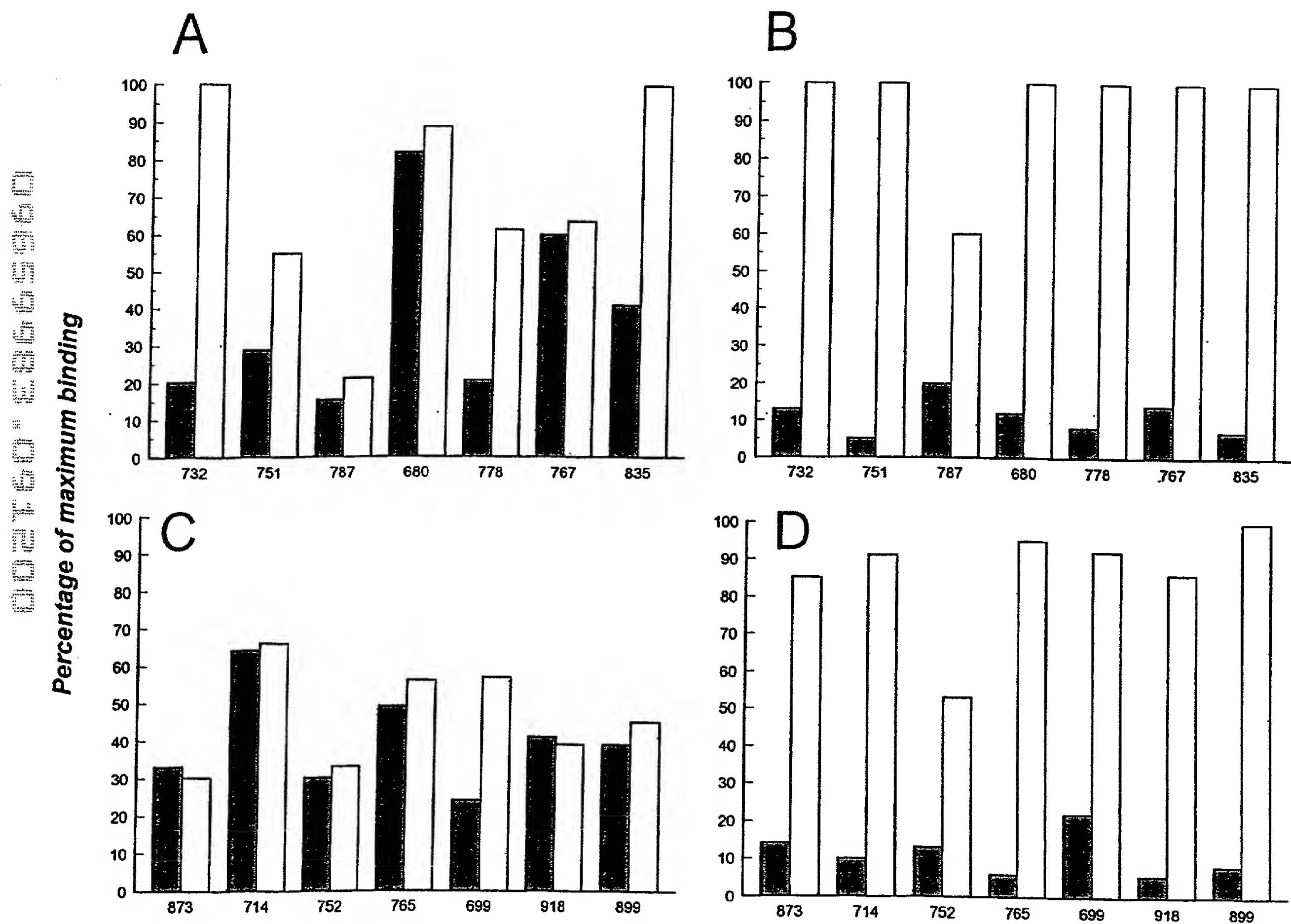
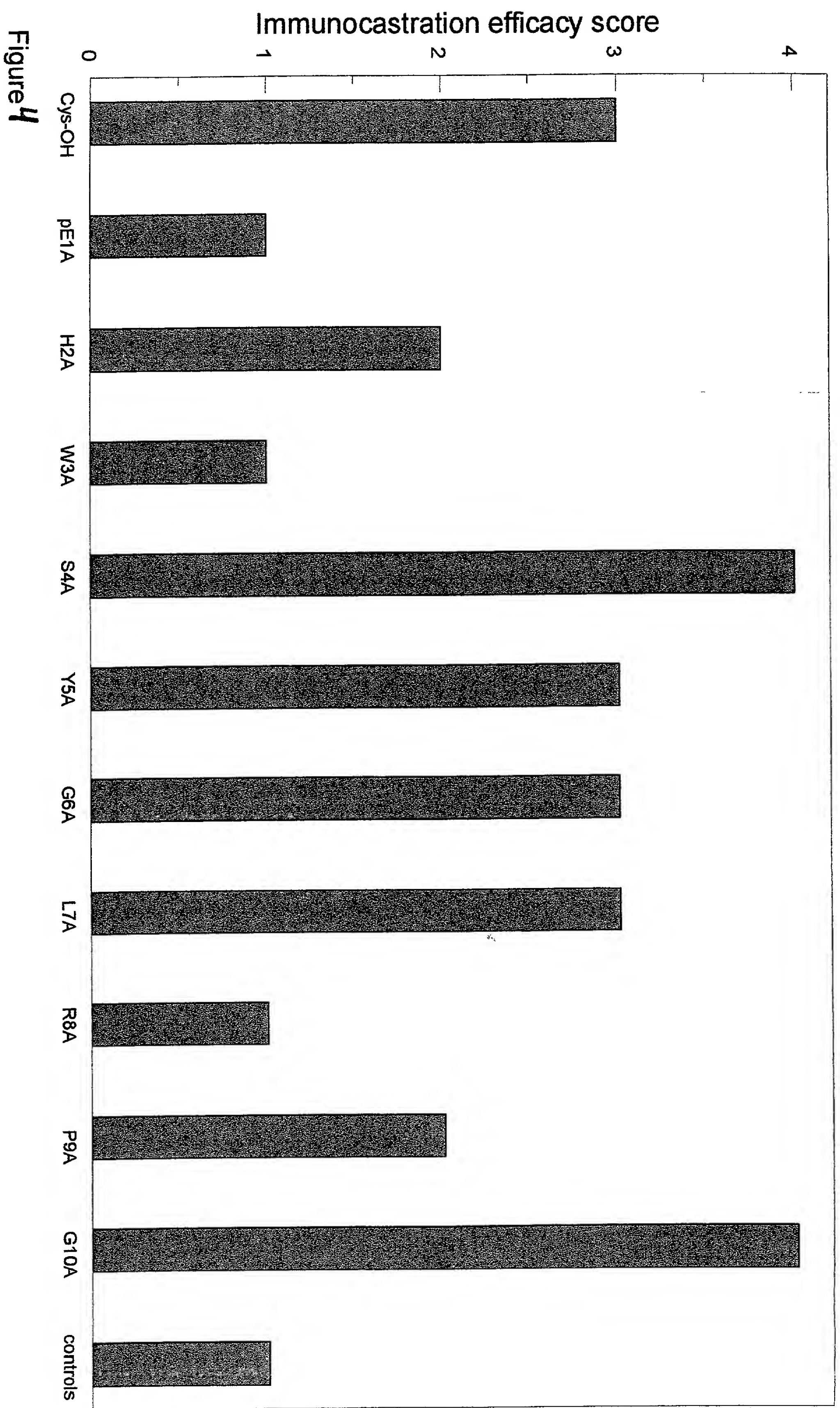


Figure 3:

GnRH Tandem Dimer Cys-OH Alascan



h/h